ORIGINAL ARTICLE

Bacillus coagulans 198 and L-glutamine in combination attenuated intestinal mucositis in a 5-FU-induced BALB/c mouse model via modulation of gut microbial community structure and diversity

Wei-Jen Chen [a,b], Siou-Ru Shen [a], Wei-Hsuan Hsu [c], Bao-Hong Lee [d], Yu-Shan Wei [a], Hui-Fang Chu [a], Ming-Chung Tseng [a], Tang-Long Shen [e,f,*]

[a] Research and Development Department, Syngen Biotech Co., Ltd., Tainan, Taiwan
[b] Graduate Institute of Management, Minghsin University of Science and Technology, Hsinchu, Taiwan
[c] Department of Food Safety/Hygiene and Risk Management, College of Medicine, National Cheng Kung University, Tainan, Taiwan
[d] Department of Horticultural Science, National Chiayi University, Chiayi, Taiwan
[e] Department of Plant Pathology and Microbiology, National Taiwan University, Taipei, Taiwan
[f] Center for Biotechnology, National Taiwan University, Taipei, Taiwan

Abstract

5-Fluorouracil (5-FU) disrupts intestinal cells and causes dysbiosis in the gut microbiota. This study explores the potential of *Bacillus coagulans*-198 (BC198) to mitigate gut microbiota imbalance and mucositis caused by 5-fluorouracil. L-glutamine is used to alleviate mucositis, and this study found that BC198 exhibits protective effects on the gut, including maintaining a healthy microbiota and reducing intestinal inflammation, regardless of whether L-glutamine is used in combination. Therefore, it can help reduce the deterioration of the gut environment caused by 5-fluorouracil. BC198 can be provided to cancer patients to prevent severe side effects, thereby improving their treatment outcomes and nutritional status.

Keywords: Bacillus coagulans-198 (BC198), 5-Fluorouracil (5-FU), Intestinal inflammation, Microbiota, Mucositis

1. Introduction

Chemotherapy often causes numerous side effects in patients, including weight loss, diarrhea, poor nutrient absorption, and anorexia. These symptoms are associated with chemotherapy-induced intestinal mucositis and the deterioration of the gut microenvironment. This not only affects the patient's quality of life but also interferes with the effectiveness of cancer treatment [1]. Significant loss of intestinal crypt cells and atrophy of intestinal villi were found in mucositis, leading to deterioration of intestinal environment. During the onset of intestinal mucositis, reactive oxygen species and inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) are produced. Studies have found that 5-FU disrupts intestinal function, affecting both the host and microbiota through the influence of inflammatory cytokines and oxidative stress [2,3].

L-glutamine (L-gln) is an important non-essential amino acid and a precursor for the biosynthesis of adenosine 5′-triphosphate (ATP). L-gln can be converted by intestinal enterocytes into various amino acids, such as proline, arginine, citrulline, and alanine, which play crucial supportive roles in the healing process of intestinal mucosal cells. Especially under stress conditions or during chemotherapy,

Received 28 June 2024; accepted 14 October 2024.
Available online 31 March 2025

* Corresponding author.
E-mail addresses: chen.william@standard.com.tw (W.-J. Chen), hsiuru@gmail.com (S.-R. Shen), whhsu@mail.ncku.edu.tw (W.-H. Hsu), bhhee@mail.ncyu.edu.tw (B.-H. Lee), Wei.vicky@syngen.com.tw (Y.-S. Wei), ChuHF@syngen.com.tw (H.-F. Chu), Tseng.albert@syngen.com.tw (M.-C. Tseng), shentl@ntu.edu.tw (T.-L. Shen).

https://doi.org/10.38212/2224-6614.3535

the gut's ability to absorb L-gln is positively enhanced. Once inside the intestinal cells, L-gln activates the gut's defense mechanisms and improves the increased permeability caused by 5-FU. Various studies have shown that L-gln can shorten the duration of chemotherapy-induced diarrhea [4–7]. Nevertheless, inconsistent results have indicated that L-gln failed to resist doxifluridine-induced diarrhea or the severity of stomatitis, nausea, and diarrhea caused by 5-FU/calcium folinate chemotherapy [8].

Probiotics such as *Lactobacillus*, *Bifidobacterium*, and *Bacillus subtilis* have been used in the development of health foods and medical supplements [9]. Compared to *Lactobacillus and Bifidobacterium*, *Bacillus* species can adapt to extreme environments (such as gastric acid, pancreatic enzymes, digestive enzymes, and bile salts) through spore formation [10–12]. *Bacillus coagulans* is a Gram-positive bacterium that produces lactic acid metabolites. After spore germination, it begins to secrete bacteriocins such as coagulin and lactosporin, and can modulate the gut microbiota and gut metabolome to enhance intestinal immune activity [13]. Recent studies have found that *B. coagulans* can reduce the number of *Escherichia coli* in the gut and increase the quantity of *Lactobacillus* [14], suggesting that *B. coagulans* could improve the gut environment, diarrhea, and symptoms associated with irritable bowel syndrome [15].

A healthy and unique gut microbiome is closely related to overall health, including nutrient provision, protection against pathogens, epithelial mucosal homeostasis, and immune system regulation [16]. In the gut, commensal microorganisms maintain dynamic balance through host regulation, and the microbial metabolites in this environment help intestinal enterocytes with renewal and repair [17]. When the dynamic balance of this unique microbiota is disrupted, it can disturb the gut microenvironment and lead to intestinal inflammation. In addition to damaging host intestinal cells, 5-FU can also cause dysbiosis and exacerbate intestinal inflammation. Clinical studies have found that after administering 5-FU, *E. coli*, *Clostridium* species, and *Enterococcus* species become dominant in the gut, replacing the original *Bifidobacterium* and *Lactobacillus* species [18].

Chemotherapy damages the intestinal function in host, leading to dysbiosis and reduction of beneficial bacteria, ultimately promoting diarrhea; in this environment, exogenous pathogenic microorganisms (such as *Shigella*) can more easily invade the gut and exacerbate diarrhea. The use of antibiotics can remove these dysbiotic microorganisms, thereby improving intestinal inflammation caused by 5-FU [18–20]. Probiotics not only help prevent mucositis but also interact with tight junction proteins on intestinal cells to facilitate colonization, enhancing mucosal integrity and eliminating the adhesion of pathogenic bacteria. The aim of this study is to investigate the protective effects of *B. coagulans* 198 (BC198) against 5-FU-induced mucositis.

2. Materials and methods

2.1. Preparation of 5-FU, L-gln, and BC198

L-gln and 5-FU were purchased from Sigma–Aldrich (MO, USA). 5-FU (5 mg/mL) and L-gln (1 g/mL) were dissolved in phosphate-buffered saline (PBS) and filtrated using syringe filter (a 0.2 μm). BC198 was obtained from Syngen Biotech Co., Ltd. (Tainan, Taiwan). BC198 was diluted in sterile water and administered via oral gavage. Mice were administered 100 μL of a suspension containing 5×10^8 CFU in deionized water daily for 17 days as the scheme shown in Fig. 1A.

2.2. Animals

Five-week-old male BALB/cByJNarl (BALB/c) mice (body weight ranging: 26.7 g–32.6 g) were purchased from the National Laboratory Animal Centre (Taipei, Taiwan). The mice were housed in a climate-controlled environment (23 ± 2 °C, relative humidity of 50 ± 5%), under a 12 h light–dark cycle, and allowed free access to food and water *ad libitum*. The mice were allowed to adapt to the environment for two weeks before the experiment. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of National Taiwan University (IACUC: NTU-104-EL-00073).

2.3. In vivo intestinal mucositis model

The mice were randomized to one of four groups (n = 6/each group): the control group, the 5-FU-induced intestinal mucositis group, the 5-FU + BC198 (5×10^8 CFU/day) group, and the 5-FU + BC198+L-gln group. Mice in all groups were given fresh sterilized water was provided each day (100 mL/daily). Intestinal mucositis was induced on days 11^{th}–13^{th} via intraperitoneal injection of 5-FU (50 mg/kg/day) in the 5-FU, 5-FU + BC198, and 5-FU + BC198 + L-gln groups. PBS was used for the control group. Mice were euthanized on day 18^{th}.

Disease severity was assessed daily via measuring body weight and diarrhea status. Diarrhea status was

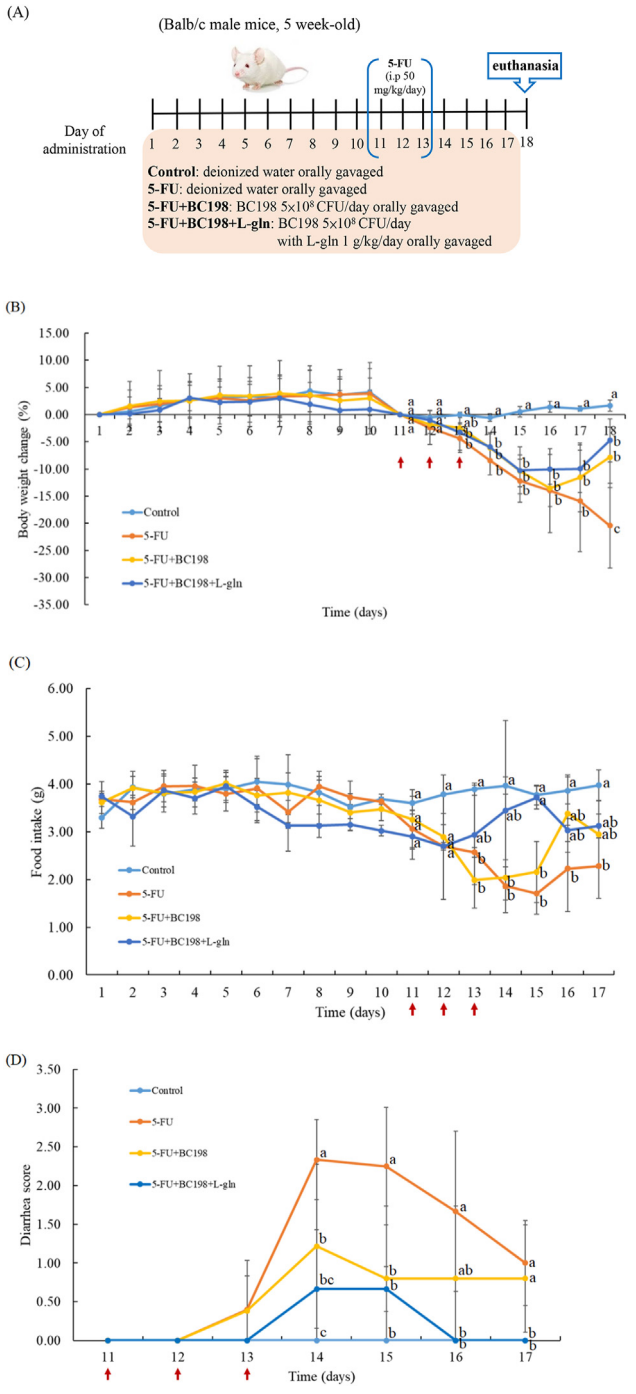


Fig. 1. Potential effect of BC198 and L-gln in attenuating intestinal mucositis in the 5-FU-induced mouse model. (A) Schematic depiction of the 5-FU-induced intestinal mucositis mouse model. 5-FU was intraperitoneally injection at the concentration of 50 mg/kg/day during 11th-13th day to induce intestinal mucositis except the mice in the control group were intraperitoneally inject PBS. All treatments were orally gavaged starting from day 1st to 17th before euthanasia. Six mice in each experimental group; (B) Body weight is shown as a percentage of initial body weight; (C) Food intake; (D) Severity of diarrhea is scored using the four-grade scale. Control: deionized water orally gavaged and PBS intraperitoneally. 5-FU: deionized water orally gavaged and 5-FU intraperitoneally. 5-FU + BC198: BC198 5 × 10^8 CFU/day orally gavaged and 5-FU intraperitoneally. 5-FU + BC198 + L-gln: BC198 5 × 10^8 CFU/day with L-gln 1 g/kg/day orally gavaged and 5-FU intraperitoneally.

graded based on stool consistency: a score of "0" indicated normal, including normal or absent stool; "1" indicated slight, including slightly wet and soft stool; "2" indicated moderate, including wet and unformed stool with moderate perianal staining of the coat; and "3" indicated severe, such as watery stool with severe perianal staining of the coat [15,19,21]. In addition, the feces were collected and stored at −80 °C for further microbiome analyses. The mice were sacrificed under anesthesia to collect the entire small intestine and colon after the removal of fat tissue, and colon length was measured.

2.4. Morphology and histopathology analysis

For the assessment of histopathological changes in intestinal tissues, the small intestine and colon were first fixed in freshly prepared 10% formalin solution for dehydration after the animals were sacrificed. The tissues were subsequently embedded in paraffin, sectioned to a thickness of 3−5 μm, and stained with hematoxylin and eosin (H&E) after deparaffinization. Morphological changes and inflammatory cell infiltration in the intestine were examined under a microscope (DL882096; LW Scientific, GA, USA).

2.5. Immunohistochemical staining of the small intestine and colon

The sections were deparaffinized in xylene, rehydrated through a graded ethanol series, and subjected to antigen retrieval using microwaves. The sections were then immersed in 3% hydrogen peroxide for 15 min to inhibit endogenous peroxidase. The slides were incubated overnight at 4 °C with an anti-F4/80 antibody (1:1000 dilution) (Abcam, catalog: ab6640), followed by washing and incubation with an HRP-conjugated secondary antibody (1:2000 dilution) (The Jackson Laboratory, ME, USA) at room temperature for 1 h. After washing away excess secondary antibody, the slides were developed with diaminobenzidine and HRP, counterstained with hematoxylin, and coverslipped. F4/80-positive cells were counted using Image J® software.

2.6. Analysis of serum pro-inflammatory cytokines

Blood was collected from the heart immediately after the mice were sacrificed. For serum collection, blood was stored at room temperature for 1 h, and centrifugation (1500×*g*, 4 °C, 15 min) was carried out to obtain serum. The concentrations of serum pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in were measured using the corresponding enzyme-

linked immunosorbent assay kits (ELISA; Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions.

2.7. Analysis for gut microbiota and short-chain fatty acid (SCFA)

Fresh fecal samples were collected from the colon of mice and immediately stored at −80°C until analysis. The standard operating procedure was followed according to the CatchGene™ Stool DNA kit (CatchGene Co., Ltd., Taipei, Taiwan). Specific primers targeting the V3-V4 region of 16S rRNA were used for polymerase chain reaction (PCR) amplification with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, MA, USA) and electrophoresis on a 2% agarose gel for detection. PCR products in the range of 400−450 bp were purified using the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany) and sequencing libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, CA, USA) with index codes added. Library quality was assessed using a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific) and an Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Sequencing was performed on the Illumina HiSeq2500 platform. Diversity and statistical analyses were conducted using the QIIME script (single_rarefaction.py), based on OTUs and taxonomic information. Statistical differences in taxonomic profiles between groups were compared using the Statistical Analysis Metagenomic Profiles software v2.1.3 with Welch's *t*-test. Partial least squares discriminant analysis (PLS-DA) was performed using QIIME (Version 1.7.0) and R software (Version 2.15.3). Additionally, statistically significant biomarkers were identified using linear discriminant analysis effect size (LEfSe). The LEfSe analysis included non-parametric Kruskal−Wallis and Wilcoxon rank-sum tests to determine significant features and abundances of bacterial taxa between groups, with linear discriminant analysis (LDA) scores calculated to estimate effect sizes (threshold >4) [22].

The concentration of short-chain fatty acids (SCFAs), including acetic acid, propionic acid, and butyric acid, in intestinal feces was measured using gas chromatography-flame ionization detection (GC-FID) (GC-2010, Shimadzu Corp, Tokyo, Japan) with a capillary column (BP21 FFAP 30 m × 0.53 mm i.d., 0.50 μm film thickness, Trajan, Melbourne, Australia). Nitrogen was used as the carrier gas (splitless injection volume was 1 μL). Auxiliary gases for the flame ionization detector were hydrogen (30 mL/min flow rate) and dry air (300 mL/min flow rate). The injector and detector temperatures were set at 220 °C and 240 °C, respectively. The GC oven temperature was initially set at 90 °C for 1 min, then increased to 150 °C at a rate of 10 °C/min, followed by an increase to 200 °C at a rate of 20 °C/min, and finally held at 200 °C for 1 min, as described in recent studies [23].

2.8. Statistical analysis

All data were analyzed using GraphPad Prism 6.0 software and presented as means ± SD for the indicated number of independent experiments. Statistical significance between experimental groups was determined via one-way analysis of variance (ANOVA) using SPSS for Windows (version 12.0), followed by Duncan's multiple range method.

3. Results

3.1. Combination of BC198 and L-gln attenuate body weight loss, food intake, and diarrhea in the 5-FU-induced intestinal mucositis model

To assess if combined supplementation of L-gln and BC198 enables effectively attenuating intestinal mucositis, we first observed the clinical symptoms, including weight change, food intake, and diarrhea using a 5-FU-induced intestinal mucositis mouse model Fig. 1A. Repeated administration of 5-FU in mice caused body weight loss, reduced food intake, and diarrhea. Compared to the control group, gradual body weight loss was observed in mice challenged with 5-FU, reaching 20.42% of the initial body weight 18 days after the challenge. In contrast, our results showed that the 5-FU + BC198, and 5-FU + BC198 + L-gln groups clearly ameliorated the 5-FU-induced impairment in body weight, resulting in moderate reductions in body weight loss by 7.85%, and 4.74%, respectively ($p < 0.05$) (Fig. 1B). In addition, the food intake was reversibly improved in the 5-FU + BC198 and 5-FU + BC198 + L-gln groups compared to the 5-FU-treated group (Fig. 1C). The diarrhea score was significantly reduced in the 5-FU + BC198, and 5-FU + BC198 + L-gln groups compared to the 5-FU-induced group, in particular on day 14th and 15th (Fig. 1D). Collectively, these data clearly showed the potential protective effect of BC198 and L-gln, separately and in combination against 5-FU-induced intestinal mucositis in a mouse model.

3.2. Effects of BC198 alone or in combination with L-gln on 5-FU-induced intestinal mucositis

In agreement with the above observations, a prominent reduction in colon length was also

observed in the 5-FU group (~7.7 cm) compared to the control group (~8.4 cm; $p < 0.05$). The shortening of colon length was moderately to significantly rescued upon treatments with BC198 alone (~8.1 cm) or in combination with L-gln (~9.3 cm; $p < 0.05$), respectively (Fig. 2A). Moreover, histological analysis with H&E staining revealed impaired mucosal epithelia and disrupted crypt-villus structures in the small intestine and colon of the 5-FU group (Fig. 2B and C) compared to the control group. In contrast, BC198 alone and in combination with L-gln significantly attenuated the histopathological abnormality.

3.3. BC198 alone or in combination with L-gln suppressed pro-inflammatory cytokine expressions in the 5-FU-induced intestinal mucositis

To further investigate mechanistic nature of the BC198 alone and combined application of L-gln and BC198 in alleviating intestinal mucositis, inflammatory factors were subjected for detection via ELISA. As illustrated in Fig. 3A–C, the serum pro-inflammatory cytokines in the 5-FU induced mice (TNF-α, 20.74 pg/mL; IL-1β, 130.16 pg/mL; IL-6, 694.00 pg/mL) were obviously increased compared to the control group (TNF-α, 0.93 pg/mL; IL-1β, 54.15 pg/mL; IL-6, 41.66 pg/mL), implicating that BC198 or in combination with L-gln could exert the anti-inflammatory ability to attenuate 5-FU induced intestinal mucositis. In agreement with the above results, we also found that BC198 or in combination with L-gln enabled resulting in significant decreases of macrophage infiltration in the small intestine and colon in comparison with those in the 5-FU-induced mice (Fig. 3D and E).

3.4. Influence on fecal microbiota community compositions by BC198 or in combination with L-gln on 5-FU-induced intestinal mucositis mice

A PLS-DA plot was constructed based on the OTU levels to evaluate the variation in the gut microbiota composition among the groups. The PLS-DA plot indicated that the variation in gut microbiota composition, respectively (Fig. 4A). The results revealed that chemotherapy caused shifts in gut microbiota composition compared to the control group. The gut microbiota compositions of the 5-FU + BC198 groups were found to be moderately different from that of the 5-FU group. On the other hand, significant changes were observed in the gut microbiota composition of the 5-F + BC198 + L-gln group compared to that in the 5-FU group. Biomarker analysis was performed using LDA, LEfSe, and a cladogram to explore the specific bacterial taxa characterized in each group. As shown in Fig. 4B, discriminative features were identified with an LDA score >4.0. The relative abundances of significantly different species ($p < 0.05$) are shown in the LEfSe taxonomic cladogram (Fig. 4C). LEfSe analysis indicated that the mucosa-associated inflammation-promoting bacteria, in particular the diarrhea-inducing bacteria *Escherichia shigella*, were increased in the 5-FU group; on the other hand, the reutericyclin-producing *Lactobacillus reuteri* was enriched in the control group; *Bacteroides thetaiotaomicron*, an acetate producer, was significantly increased in the 5-FU + BC198 group; the anti-inflammatory strain *Lactobacillus murinus* was elevated in the 5-FU + BC198 + L-gln group.

Next, the detail of gut microbiota composition in fecal samples was evaluated. Taxonomic analysis at the phylum level revealed that 5-FU altered the gut microbiota composition (Fig. 5A). The proportion of *Proteobacteria* in the 5-FU group was 0.129, higher than that in the control group (0.026). Supplementation with BC198 increased the abundance of *Proteobacteria* to 0.038. Surprisingly, the combination of BC198 and L-gln led to a reduced distribution pattern of 0.007. These results highlight the intestinal microecological changes upon 5-FU treatment; however, oral administration of BC198 alone or in combination with L-gln differentially and specifically altered the 5-FU-altered gut microbiota, and further obtaining a genus-level abundance cluster heatmap (Fig. 5B). First of all, we revealed that the abundance of *Muribaculaceae* in the 5-FU and 5-FU + BC198 groups were 34.59% and 33.11%, respectively, a great reduction than that observed in the control group (52.57%). Oral supplementation with BC198 increased the abundance of *Muribaculaceae* to 39.96%. As shown in the tree-map results, the abundance of *Muribaculaceae* in the 5-FU + BC198 + L-gln group was 47.33%, higher than that in the groups treated the 5-FU or 5-FU + BC198 group. Moreover, *Ruminococcaceae*_UCG_014, *Ruminococcaceae*_UCG_013, *Candidatus Saccharimonas,* and *Enterorhabdus* were increased in the control group; however, *E. shigella, Enterococcus, Odoribacter, Acinetobacter, Alistipes*, and *Lachnospiraceae*_UCG_006 were increased in the 5-FU group. Additionally, *Ruminiclostridium, Blautia, Anaerotruncus, Butyricicoccus, Akkermansia*, and *Parasutterella* were increased in the 5-FU + BC198 group; *Ruminiclostridium*_5, *Lachnoclostridium, Ruminiclostridium*_6, the *Eubacterium coprostanolilgenes*, and *Ruminiclostridium*_9 were increased in the 5-FU + BC198 + L-gln group. These results indicated that the abundance of some intestinal microbes

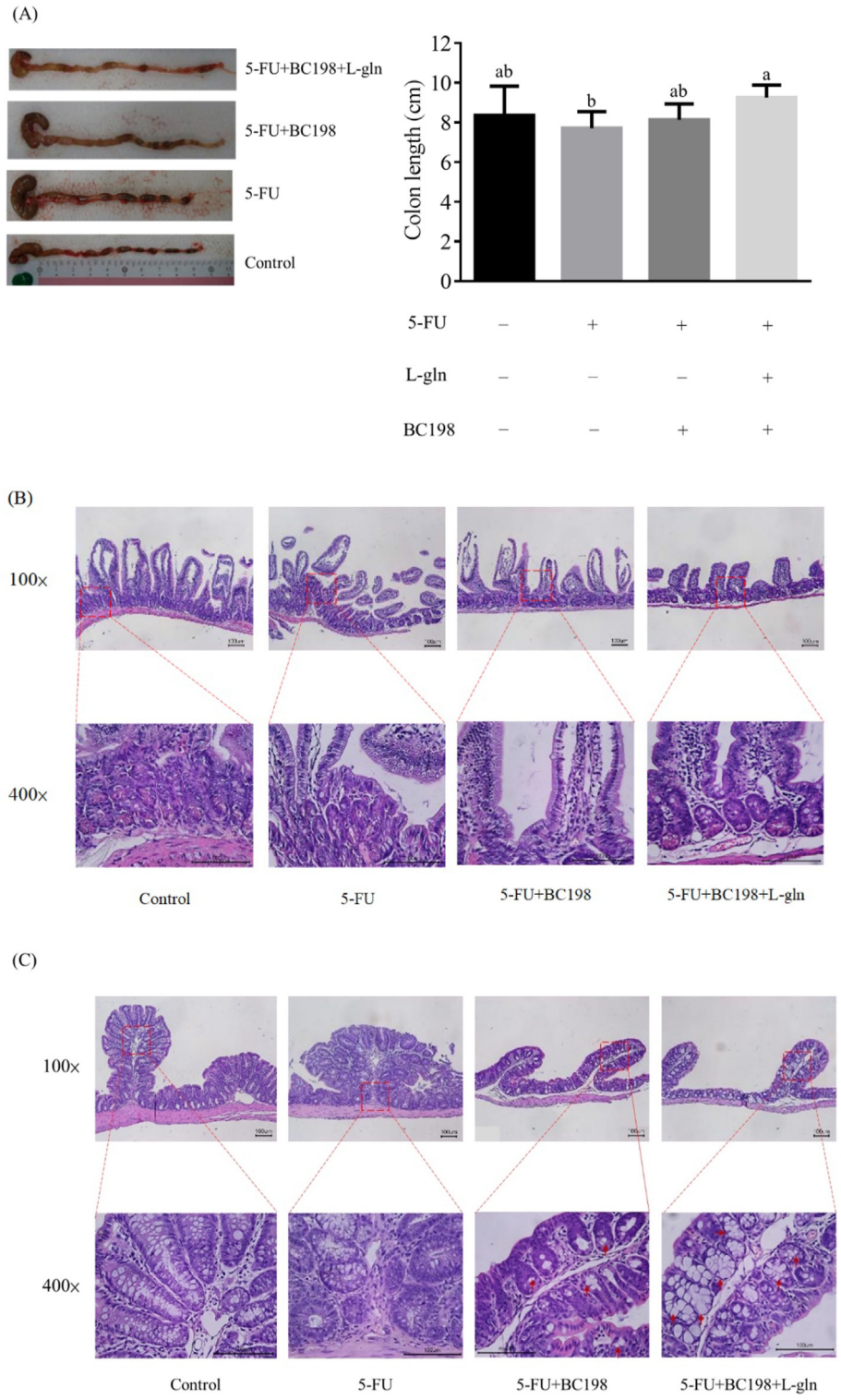


Fig. 2. Histopathology of BC198 and L-gln treatments on 5-FU-induced intestinal mucositis. Four groups of mice as indicated were sacrificed as per the experimental design and subjected to measurement of (A) colon length; hematoxylin and eosin staining of the (B) small intestine and (C) colon after excision and sectioning. The representative images (100× and 400× magnifications) of each group are presented. Data are presented as mean ± SD ($n = 6$). The data with different superscripted letters are significantly different based on the one-way ANOVA ($p < 0.05$).

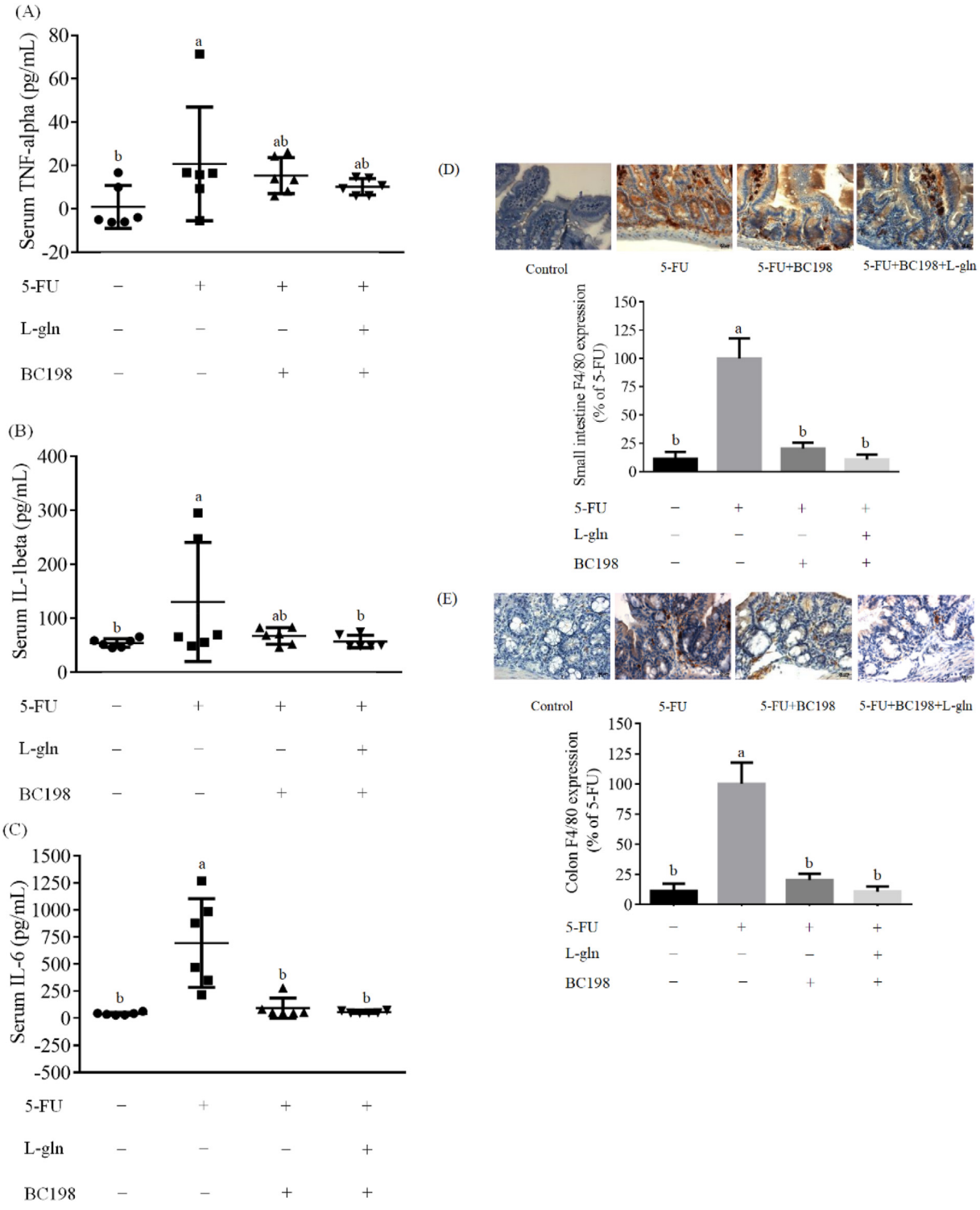
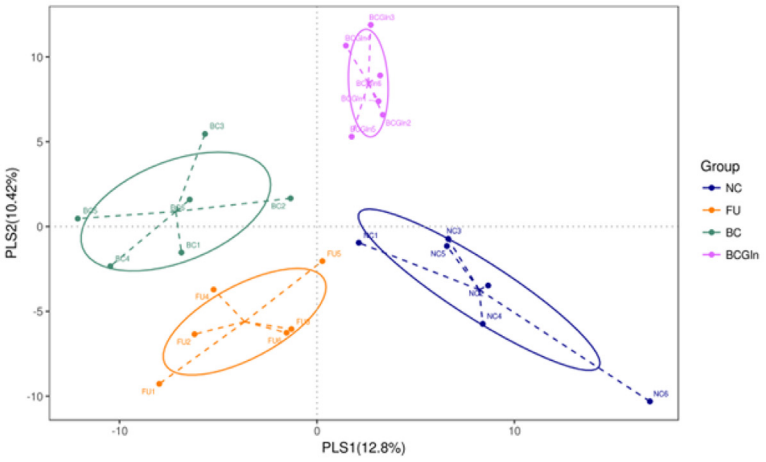
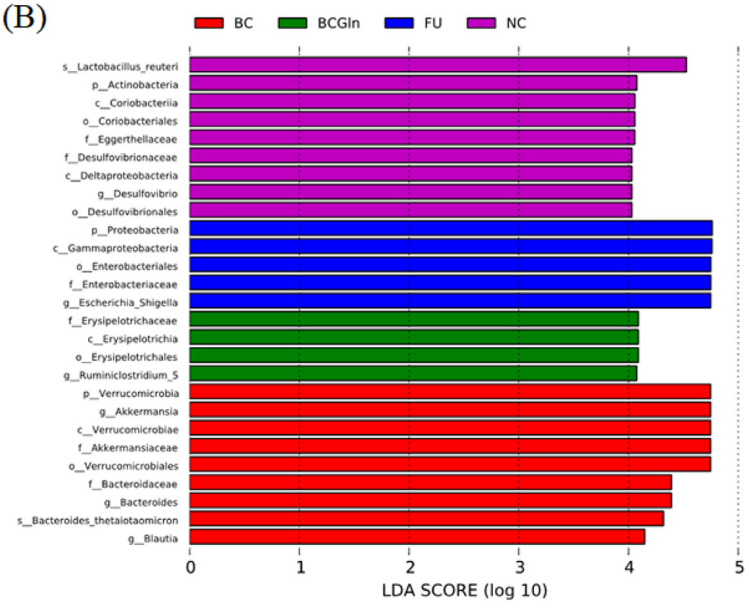


Fig. 3. Reduction of the inflammatory effect via treatment of BC198 or in combination with L-gln in the 5-FU-induced intestinal mucositis mouse model. The elevated expressions of TNF-α (A), IL-1β (B), IL-6 (C), and F4/80 (a marker for macrophage, D and E) induced by 5-FU were significantly diminished by the treatment of BC198 or in combination with L-gln in serum (A−C) or in the intestine (D) and the colon (E) in the 5-FU-induced intestinal mucositis mice. The representative images (100× and 400× magnifications) of each group are presented. Data are presented as mean ± SD (n = 6). Data with different superscripted letters are significantly different based on the one-way ANOVA (p < 0.05).

(A)


PLS2(10.42%)
PLS1(12.8%)
Group
NC
FU
BC
BCGln

(B)


BC
BCGln
FU
NC
s__Lactobacillus_reuteri
p__Actinobacteria
c__Coriobacteriia
o__Coriobacteriales
f__Eggerthellaceae
f__Desulfovibrionaceae
c__Deltaproteobacteria
g__Desulfovibrio
o__Desulfovibrionales
p__Proteobacteria
c__Gammaproteobacteria
o__Enterobacteriales
f__Enterobacteriaceae
g__Escherichia_Shigella
f__Erysipelotrichaceae
c__Erysipelotrichia
o__Erysipelotrichales
g__Ruminiclostridium_5
p__Verrucomicrobia
g__Akkermansia
c__Verrucomicrobiae
f__Akkermansiaceae
o__Verrucomicrobiales
f__Bacteroidaceae
g__Bacteroides
s__Bacteroides_thetaiotaomicron
g__Blautia
LDA SCORE (log 10)

(C)

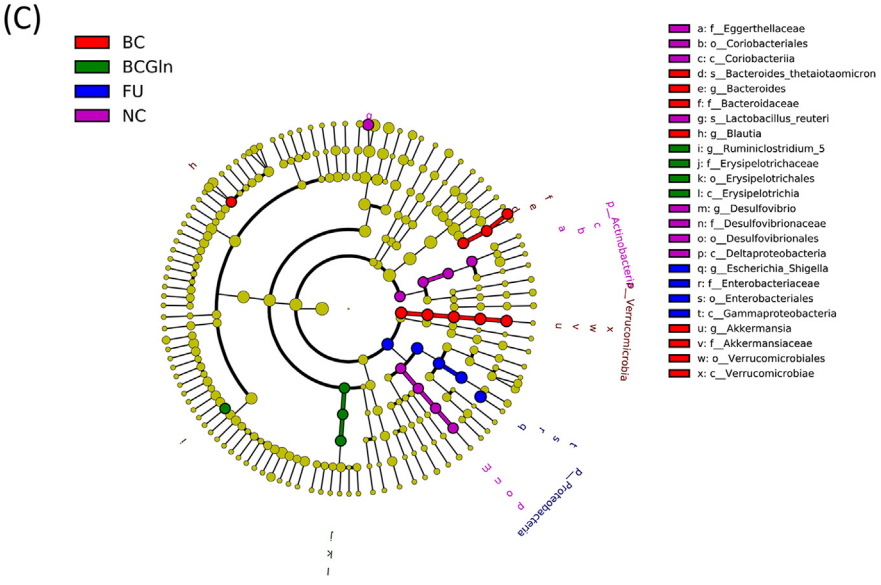

BC
BCGln
FU
NC
a: f__Eggerthellaceae
b: o__Coriobacteriales
c: c__Coriobacteriia
d: s__Bacteroides_thetaiotaomicron
e: g__Bacteroides
f: f__Bacteroidaceae
g: s__Lactobacillus_reuteri
h: g__Blautia
i: g__Ruminiclostridium_5
j: f__Erysipelotrichaceae
k: o__Erysipelotrichales
l: c__Erysipelotrichia
m: g__Desulfovibrio
n: f__Desulfovibrionaceae
o: o__Desulfovibrionales
p: c__Deltaproteobacteria
q: g__Escherichia_Shigella
r: f__Enterobacteriaceae
s: o__Enterobacteriales
t: c__Gammaproteobacteria
u: g__Akkermansia
v: f__Akkermansiaceae
w: o__Verrucomicrobiales
x: c__Verrucomicrobiae
p__Actinobacteria
p__Verrucomicrobia
p__Proteobacteria

Fig. 4. Influence of gut microbiota compositions by BC198 or in combination with L-gln on 5-FU-induced intestinal mucositis mice. Partial least squares discriminant analysis (PLS-DA) of gut microbiota communities based on operational taxonomic unit (OTU) levels (A). Linear discriminant analysis effect size (LEfSe) was used to compare the differences in microbial abundance among different groups (B). The cladogram displays the composition and proportion of microorganisms at different taxonomic levels. The innermost layer shows the taxonomic tree. The circle from the inside to outside represents different taxon levels from phylum to genus. Yellow circles represent no statistical difference in species among different groups, and red circles, green circles, blue circles, purple circles, and the green lake represent higher abundance of genera among different groups. Lowercased letters corresponding to different bacteria are shown in the legends (C).

shifted as a result of BC198 or in combination with L-gln treatment. Moreover, we also found that *Lactobacillus* population was greater reduced in mice treated with 5-FU at genus level, which could be recovered by BC198 or in combination with L-gln in the 5-FU induced mice (Fig. 5C).

3.5. Elevation of specific SCFA productions by BC198 and L-gln treatments in association with the recovery of the 5-FU-induced gut dysbiosis

SCFAs (short chain fatty acids) affect the absorption and metabolism in the host [24]. As shown in Table 1, the administration of BC198 (134.8 mmol/g) or in combination with L-gln (165.6 mmol/g) significantly increased total short chain fatty acids (SCFAs) in the fecal, particularly acetic acid and butyric acid, compared to the 5-FU group (111.0 mmol/g) in the 5-FU-induced intestinal mucositis mice. This finding is in accordance with the above improvements in intestinal functions (Figs. 2 and 3) as well as the elevations of *Ruminiclostridium*_5 and *Lactobacillus* according to the Welch's *t*-test analysis while the 5-FU induced mice treated with BC198 or BC198 + L-gln, respectively (Fig. 6) (see Fig. 7).

4. Discussion

L-gln has been anticipated as a promising adjunct compound for chemotherapy due to its ability to improve mucositis induced by radiotherapy and chemotherapy, and to support the nutritional absorption efficiency of intestinal enterocytes [25]. When nutritional absorption is affected by intestinal mucositis, it can reduce the turnover of crypt cells and lead to a decrease in the absorptive surface area of the villi, ultimately causing weight loss [26]. These phenomena are closely associated with the negative effects of chemotherapy-induced intestinal mucositis [27]. Typical features of intestinal mucositis include the destruction of intestinal structure (villus breakage and atrophy, loss of crypt structure, increased goblet cell depletion, and infiltration of inflammatory cells) [28,29]. When epithelial cells in the intestinal crypt undergo apoptosis, the intestinal epithelial barrier is subsequently compromised, leading to continuous bacterial translocation within the lumen [30]. 5-FU has been shown to destroy mucosal integrity, reduce villus and crypt depths, and increase inflammation severity, resulting in side effects such as nausea, diarrhea, anorexia, and weight loss [31]. As shown in Fig. 1, significant reductions in body weight and food intake, along with severe diarrhea, were observed after the induction of intestinal mucositis in the 5-FU group. Administration of BC198 or in combination with L-gln significantly alleviated weight loss (Fig. 1B–D). These results indicate that BC198 and L-gln can mitigate weight loss, reduced food intake, and diarrhea caused by intestinal inflammation. We also found that the induction of intestinal mucositis led to increased damage to the villi in the small intestine and loss of crypt structure in the colon segments. However, administration of BC198 or in combination with L-gln significantly enhanced the villus and crypt structures in the intestine. These results suggest that the combined therapy with BC198 and L-gln helps to alleviate intestinal damage (Fig. 2).

Pro-inflammatory cytokines have been shown to play a pivotal role in amplifying the severity of chemotherapy-induced intestinal mucositis [32], and it has also been reported that 5-FU treatment increases serum pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) [27]. We found that the production of these pro-inflammatory cytokines can be reduced by treatment with BC198 or in combination with L-gln (Fig. 3A–C). The gut microbiota is a crucial regulator of health, and its dysbiosis can impact intestinal tissue by introducing invasive antigens, activating immune cells, and producing inflammatory cytokines [33]. The toxicity of chemotherapeutic agents can disrupt the survival of beneficial gut bacteria and lead to intestinal cell death, with substances released from damaged cells promoting the growth of harmful bacteria associated with intestinal mucositis [34]. A study indicated that fermented-product could improve microbiota *in vitro* [35,36]. In this study, mice with 5-FU-induced intestinal mucositis exhibited dysbiosis and disrupted metabolic activity of the gut microbiota, but BC198 could improve intestinal bacteria flora (Fig. 4).

Based on OTUs analysis, 5-FU treatment significantly increased the relative abundance of Proteobacteria at the phylum level (Fig. 5). Proteobacteria

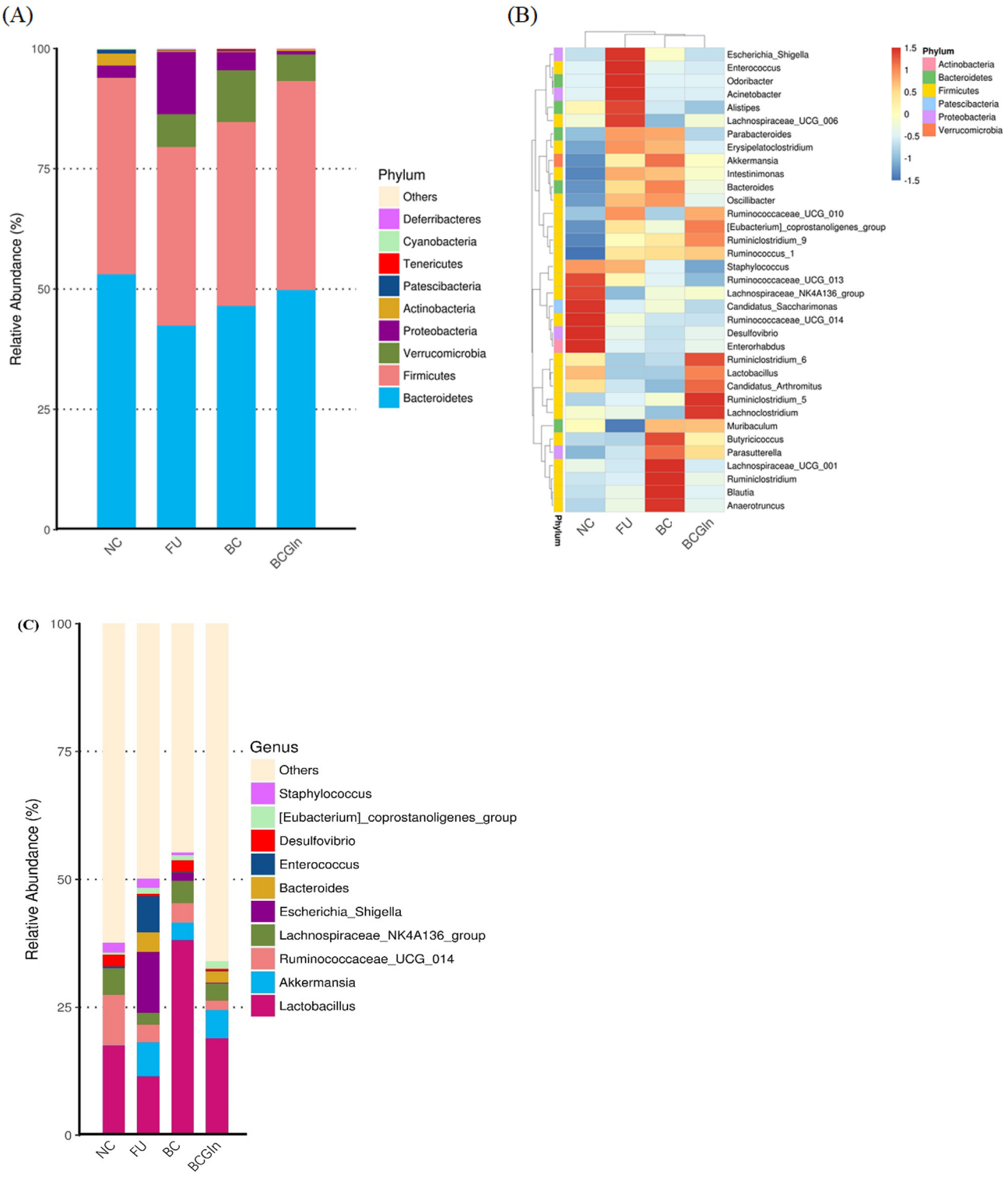


Fig. 5. BC198 alone or in combination with L-gln altered the taxonomic composition of fecal bacterial communities. (A) Top 10 species at the phylum level (relative abundance), (B) microbiota at genus level via Heatmap, and (C) Top 10 species at the genus level (relative abundance).

produce lipopolysaccharides (LPS), which can increase intestinal permeability, subsequently activates inflammatory signals, exacerbating the symptoms of intestinal mucositis [37].

5-FU increases the abundance of Enterobacteriaceae in the gut, a family of Gram-negative bacteria belonging to the Proteobacteria phylum. This family includes many known gut pathogens, such as *E. coli*

Table 1. Combination of B. coagulans and L-gln modulate SCFAs production in 5-FU-induced intestinal mucositis.

Groups	Acetic acid	Propionic acid	Butyric acid	Total SCFAs
	mmol/g (fecal content)			
Control	68.2 ± 11.3[b]	17.4 ± 6.4	18.3 ± 4.3	103.9 ± 3.7[b]
5-FU	75.5 ± 8.8[b]	16.2 ± 4.2	19.3 ± 5.0	111.0 ± 12.3[b]
5-FU + BC198	94.1 ± 15.0[a]	15.9 ± 8.0	24.8 ± 15.1	134.8 ± 14.3[ab]
5-FU + BC198 + L-gln	118.7 ± 20.9[a]	16.2 ± 6.0	30.8 ± 10.6	165.6 ± 23.1[a]

Control: deionized water orally gavaged + phosphate-buffered saline intraperitoneally. 5-FU: deionized water orally gavaged + 5-FU intraperitoneally. 5-FU + BC198: BC198 5 × 10^8 CFU/day orally gavaged + 5-FU intraperitoneally. 5-FU + BC198 + L-gln: BC198 5 × 10^8 CFU/day with L-gln 1 g/kg/day orally gavaged + 5-FU intraperitoneally. Significant difference was shown by various superscript letters ($p < 0.05$).

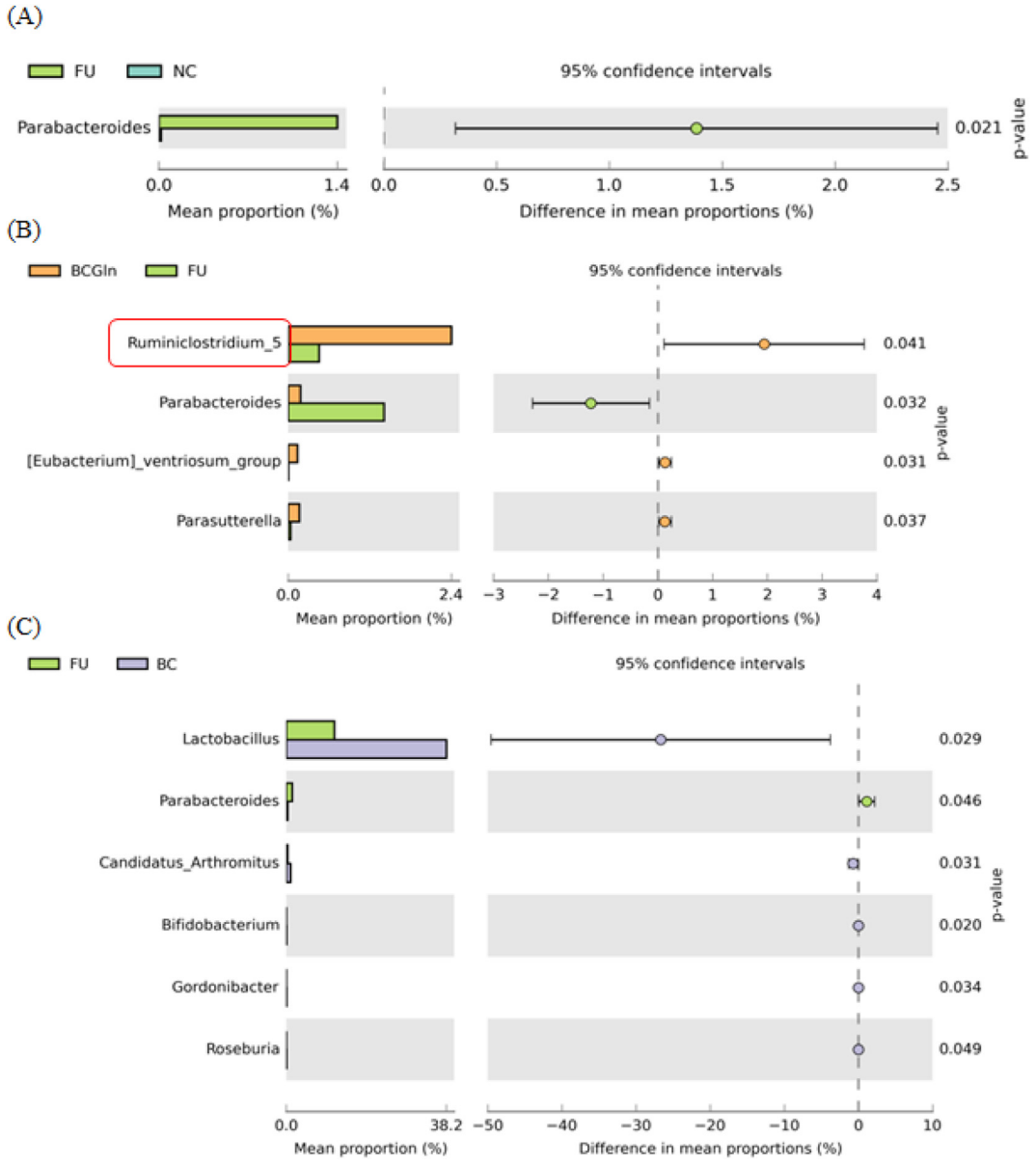


Fig. 6. Differential species between two groups. (A) Welch's t-test between NC and 5-FU. (B) Welch's t-test between 5-FU and BC198. (C) Welch's t-test between 5-FU and BC198+L-gln. The left chart represents the mean abundance ratio of different significantly species in the two groups. The right chart represents the confidence of the difference between groups. The leftmost endpoint of each circle in the graph represents the lower bound of the 95% confidence interval for the mean difference, and the rightmost endpoint represents the upper bound. The center of the circle represents the difference between the mean values. The group with high mean value would be represented by the circle color. The value on the right is the p-value of the significance test between groups of different species, and $p < 0.05$ indicates the difference.

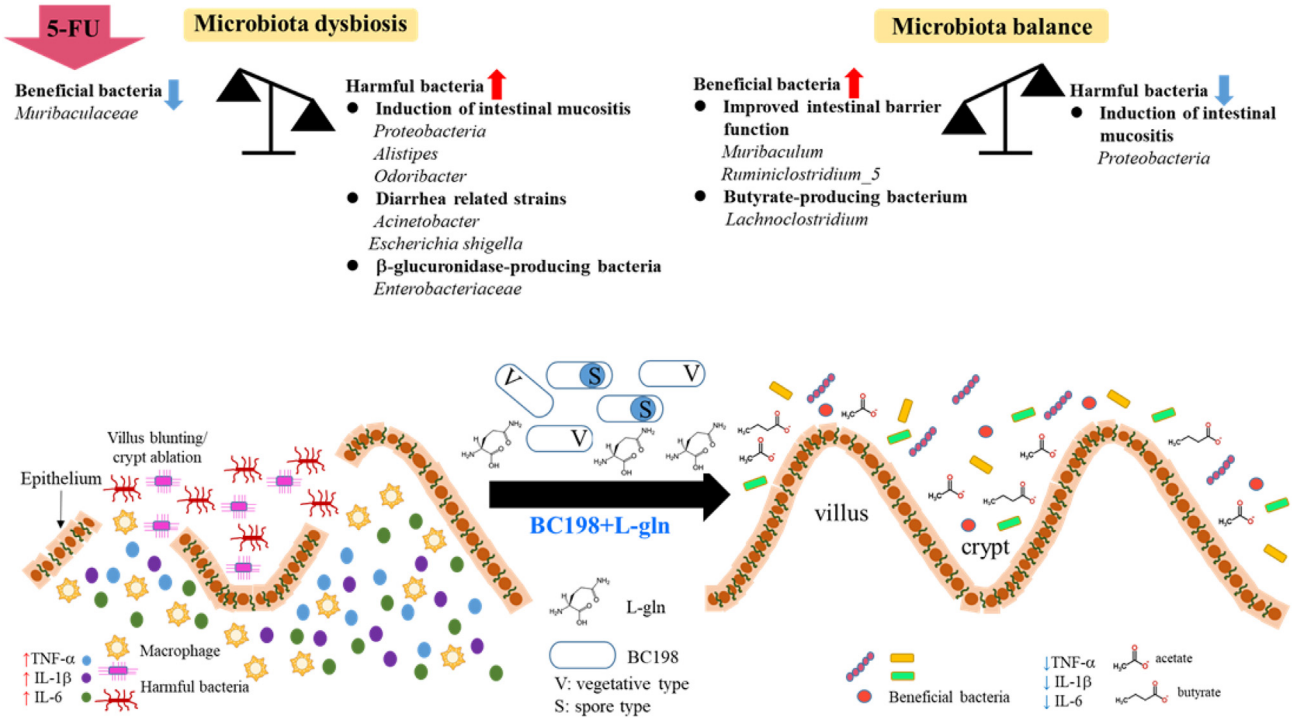


Fig. 7. A schematic model for BC198 or in combination with L-gln attenuating 5-FU-induced intestinal mucositis. Modulation of gut microbial community structure and diversity in the intestine enables reduction in inflammation and maintenance of barrier function in intestine and colon, improving the intestinal mucositis symptoms.

and *Shigella* [38], which showed increased relative proportions in the 5-FU group in our study (Fig. 5C). In contrast, BC198 or its combination with L-gln significantly reduced the abundance of these bacteria (Fig. 5C). Previous research has shown that metabolites from colonic inflammation can selectively promote the growth of commensal *Enterobacteriaceae*, thereby contributing to gut dysbiosis, and probiotics were applied to improve inflammation [39]. Although our findings support the conclusion that chemotherapy induces gut microbiota dysbiosis, further research is needed to understand the changes in gut microbiota and their causes during intestinal mucositis.

PLS-DA can effectively distinguish observed values between groups and identify influencing variables result in differences between groups to avoid the deficiency of the principal component analysis. The beta diversity of PLS-DA revealed that the gut microbiota in the 5-FU group deviated significantly from that in the control group. Our results showed significant differences in the community compositions of the group administered BC198 or in combination with L-gln (Fig. 4A). LEfSe is a tool for biomarker discovery and explanation of differences between groups for high-dimensional data. Our results (Fig. 4B and C) clearly demonstrate an imbalance in the gut microbiota of 5-FU-treated mice due to a significant increase in the abundance of *Enterobacteriaceae*, and *E. shigella*.

Cancer therapy decreases the abundance of *Bifidobacterium*, *Faecalibacterium prausnitzii*, and *Clostridium* cluster XIVa in the gut, while increasing the abundance of *Bacteroides* and *Enterobacteriaceae*. These changes are strongly associated with the development of intestinal mucositis and diarrhea [40]. In our study, the main biomarkers in the 5-FU + BC198 group included p_*Verrucomicrobia*, g_*Akkermansia*, c_*Verrucomicrobiae*, f_*Bacteroidaceae*, and s_*B. thetaiotaomicron*. For the 5-FU + BC198 + L-gln group, the primary biomarkers were f_*Erysipeiotrichaceae*, c_*Erysipelotrichia*, o_*Erysipelotrichales*, and g_*Ruminiclostridium*. It has been confirmed that *B. thetaiotaomicron* can reduce NF-kappaB activation, thus exhibiting anti-inflammatory effects [41].

Many probiotics and L-gln have been shown to have protective potential against chemotherapy-induced intestinal mucositis [42–45]. In our study, 5-FU administration significantly increased the relative abundance of the glucuronidase-producing bacterium *E. shigella* and resulted in significant intestinal damage, which could be substantially ameliorated by administration of probiotics (*i.e.* BC198) and glutamate attributed by reduction of

inflammatory factors and elevation of short chain fatty acids empowering anti-inflammatory activity (Fig. 7).

5. Conclusion

This is the first study to investigate the effects of probiotics alone and in combination with L-gln on the 5-FU-induced intestinal mucositis mouse model. We conclude that oral administration of the probiotics BC198 or in combination with L-gln attenuates 5-FU-induced intestinal mucositis via modulating the gut microbial community structure and diversity in correlation with the maintenance of inflammatory homeostasis and preservation of intestinal permeability. The combination of BC198 and L-gln greatly promoted the growth of certain gut bacteria, such as the anti-inflammatory lactic acid bacteria. We believe that the data generated in this study will be of importance for the prevention and management of 5-FU-induced intestinal mucositis.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The authors would like to thank Dr. Yu-Lun Kuo at BIOTOOLS Co., Ltd in Taiwan for kindly supporting analysis of NGS data and gratefully acknowledge financial support from the Syngen Biotech Co., Ltd. in Taiwan.

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